THE EFFECTS OF VANADIUM, COPPER, MANGANESE AND IRON ON THE SYNTHESIS OF PROTOPLASM BY CHILOMONAS PARAMECIUM

by

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THE EFFECTS OF VANADIUM, COPPER, MANGANESE AND IRON ON THE SYNTHESIS OF PROTOPLASM BY CHILOMONAS PARAMECIUM [1]

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About forty years ago it was believed that only ten elements are necessary for the synthesis of living matter—carbon, oxygen, hydrogen, nitrogen, phosphorus, sulfur, calcium, magnesium, potassium and iron. It was known before then that many heavy metals besides iron are found in living matter but it has been only during recent years that their indispensability in some organisms and beneficial action in others have been demonstrated. Vanadium, copper, and manganese are among these; however, practically nothing has been done concerning the effect of these elements on the protozoa.

An excellent opportunity to make such a study is offered by the colorless biflagellate protozoan, *Chilomonas paramecium*, which can synthesize starch, fat, proteins and protoplasm from relatively simple compounds under environmental conditions which can be accurately controlled. The results presented in the following pages were obtained in a study of the effects of vanadium, copper, manganese and iron on the rate of reproduction of this organism.

Material and Methods

The original specimens of *Chilomonas paramecium* used in these experiments were obtained from a strain which has been maintained in the Zoölogical Laboratory of The Johns Hopkins University for eight years, at first, in sterile acetate-ammonium solution (Mast and Pace, 1937) and lately in a sterile modified acetate-ammonium solution (Table I). The modified acetate-ammonium solution will be referred to as the acetate-ammonium solution.

Kahlbaum chemicals "highest purity" were used exclusively. All water used in the preparation of solutions was redistilled in a tandem Pyrex glass still (Mast, 1928). Chemically clean Pyrex glass double

[1] The author acknowledges his profound appreciation to Professor S. O. Mast for invaluable criticisms and assistance in the preparation of this manuscript and to Dr. D. M. Pace for helpful advice and suggestions in the experiments.

depression slides, Erlenmeyer flasks, and pipettes were used throughout in the experimental work.

Tests were made to ascertain whether the Kahlbaum salts used to prepare the acetate-ammonium solution contained manganese and ferric iron. The test used to detect manganese depends upon the formation of $KMnO_4$ from KIO_4 and salts of manganese when together in acid solution. By this method concentrations of manganese as low as 10^{-5} M can be detected. By using concentrated solutions of salts in which manganese cannot be detected, diluted 1000 times in making the acetate-ammonium solution, it is certain that the concentration of manganese in the culture medium did not exceed 10^{-8} M.

Two tests were used for iron, one based on the formation of $Fe_4[Fe(CN)_6]_3$ (Prussian blue) by ferric iron in the presence of $K_4Fe(CN)_6$ and the other on the formation of $Fe(CNS)_6^{---}$(ferric thiocyanate ion) by ferric iron in the presence of KCNS (Feigl, 1937;

TABLE I

Composition of the modified acetate-ammonium solution used to culture *Chilomonas paramecium*. Hydrogen ion concentration adjusted to pH 6.8 by means of HCl.

Chemicals	Mgm. per 100 cc. Water	Molar Concentrations
$NaC_2H_3O_2$	150.00	0.011
K_2HPO_4	20.00	0.0011
NH_4Cl	46.00	0.0086
$(NH_4)_2SO_4$	10.00	0.00076
$CaCl_2$	1.16	0.00010
$MgCl_2$	1.00	0.00010
HCl	Variable	Variable

pp. 93 and 95). By these methods a concentration of iron as low as 5×10^{-5} M can be detected. By using concentrated solutions of salts in which iron cannot be detected, diluted 1000 times in making the acetate-ammonium solution, it is certain that the concentration of iron in the culture medium did not exceed 5×10^{-8} M. Kahlbaum contends, however, that the $CaCl_2$ used contained 0.0001 per cent iron. If this is true the concentration of iron was at least 1.98×10^{-10} M.

The effects of the heavy metals on *Chilomonas* were ascertained by comparing the growth of isolated specimens in acetate-ammonium solution to which varying amounts of compounds of heavy metals were added with the growth of specimens in acetate-ammonium solution to which none were added.

The solutions containing various amounts of heavy metals were prepared as follows: To 49.5 cc. of acetate-ammonium solution in a chemically clean 125 cc. Pyrex glass Erlenmeyer flask enough of the compound of the heavy metal in 0.5 cc. of solution was added to yield

50 cc. of solution containing the highest concentration tested. Then by serial dilution, 50 cc. of each concentration of the metal being tested was prepared in acetate-ammonium solution in individual 125 cc. Erlenmeyer flasks.

The hydrogen ion concentration of these solutions, except in the higher concentrations of the compounds of the heavy metals, was pH 7.2. Since *Chilomonas* grows best at pH 6.8 to 6.6 (Mast and Pace, 1938), enough HCl was added to increase the hydrogen ion concentration to pH 6.8. The amount of HCl needed varied with the kind and concentration of the compound of the heavy metal in the solution. The highest concentration of $VOCl_2$ required none; the highest concentration of Na_3VO_4 required 0.1 cc. 0.4 M; and the other high concentrations and all low concentrations required amounts varying between these. The hydrogen ion concentration was measured daily and maintained at pH 6.8 to 6.6 throughout each experiment.

After the solutions were prepared, the flasks were stoppered with non-absorbent cotton, heated in an oven to 80° C. and kept at this temperature for about 20 minutes. This heating was repeated daily, resulting in solutions which were always entirely free of bacteria and molds.

The experiments were performed as follows: Two dry clean double depression slides were put into each of a number of Petri dishes, heated to 135° C. and left at least 30 minutes. After cooling, each Petri dish was labeled to indicate the kind of solution which was to be put into it, and then 0.1 cc. of that solution put into each of the four depressions. (A different pipette was used for transferring each solution.) One chilomonad was then transferred to each depression in all Petri dishes, directly from a sterile clone which had been growing in acetate-ammonium solution for two or three days. The Petri dishes were then put into an incubator in which the air was very humid and the temperature 24 $\pm$ 0.5° C. After 24 hours the number of divisions that had occurred in each depression was recorded and one individual from each depression was transferred to another depression containing fresh solution of the kind from which it had been taken. The specimens which were not transferred were either discarded or stained to ascertain the amount of starch and fat in them. This procedure was repeated daily as long as the experiment was continued.

Vanadium

Vanadium is usually thought of as a rare metal. This, the evidence shows, is hardly true. It occurs in the crust of the earth to the

extent of 0.017 per cent, whereas copper, zinc, and lead occur respectively to the extent of only 0.0104, 0.0039 and 0.0020 per cent (Marks, 1929).

The occurrence of vanadium in living systems is rare, but it has been known for a long time to occur in plants. According to Suzuki (1903), Lippmann found it in 1888 in the ash of sugar beets, and Demarcay (1900) found it in the wood of oak, elm, poplar and pine trees, and the grape vine.

Henze (1932) found vanadium in 1911 in the blood corpuscles of the ascidian, *Phallusia mammillata*. Vinogradov (1930) found it in *Phallusia obliqua* and remarked that this species is so numerous along the shores of the Russian Arctic Sea that a great accumulation of vanadium now exists there. Vinogradov postulated that the metal functionally takes the place of iron and copper in the blood of the ascidians, implying that it functions in the carrying of oxygen. Henze presented evidence against this view and suggests that the divalent vanadium of the ascidian blood in some way serves as a reducing agent in the formation of the cellulose in the tunic.

Experimental Procedure

The effects of partially reduced vanadium (tetravalent), in $VOCl_2$, and of oxidized vanadium (pentavalent), in Na_3VO_4, were ascertained as follows: Varying concentrations of each were prepared in acetate-ammonium solution, the hydrogen ion concentration adjusted, the solution kept free of bacteria and molds and the experiments made as described above.

Seven experiments were made to ascertain the effect of $VOCl_2$. Of these experiments one was continued 26 days, one 40 days, two 12 days, two 14 days and one 15 days. The chilomonads used in all except one of the experiments were selected from ordinary vigorous cultures. Those used in this experiment were obtained from a culture produced by continuously selecting slow growing specimens for many generations. The former divided considerably more frequently than the latter under identical conditions.

In the former experiments the four original lines in each concentration were continued for the duration of the experiment, but in the latter new lines were started every eight days from a line that had divided regularly throughout the preceding eight days, twice in acetate-ammonium solution containing no vanadium and twice in acetate-ammonium solution containing an optimum concentration of $VOCl_2$. The latter were washed free of vanadium before transferring.

Three experiments were made to ascertain the effect of Na_3VO_4.

Two were continued 15 days and one 12 days. In each the chilomonads used were selected from ordinary vigorous cultures.

The results obtained are presented in Tables II and III and in the following paragraphs.

Results

Table II shows that in experiment I as the concentration of $VOCl_2$

TABLE II

Effect of partially oxidized vanadium (tetravalent), in $VOCl_2$, in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature, 24 ± 0.5° C.

Molar Concentrations of $VOCl_2$	Average Number Divisions per Day of Four Lines	
	Experiment I (26 days)	Experiment II (40 days)
0	3.19±.040*	2.20±.033*
10^{-7}	3.33±.054	2.39±.027
10^{-6}	3.58±.051	2.43±.029
4×10^{-6}	3.58±.064	———
5×10^{-6}	———	2.43±.029
7×10^{-6}	3.38±.063	———
10^{-5}	3.65±.041	2.42±.029
4×10^{-5}	3.67±.039	———
5×10^{-5}	———	2.54±.029
7×10^{-5}	3.61±.054	———
10^{-4}	3.33±.048	2.33±.030
3×10^{-4}	2.97±.086	———

* Probable errors.

increased the rate of reproduction in *Chilomonas* increased from 3.19 divisions per day in acetate-ammonium solution with no vanadium to 3.67 at 4×10^{-5} M and then decreased to 2.97 at 3×10^{-4} M, and that in experiment II the rate of reproduction increased from 2.20 divisions per day in acetate-ammonium solution with no vanadium to 2.54 at 5×10^{-5} M and then decreased to 2.33 at 10^{-4} M. These results show then that partially reduced vanadium (tetravalent), in $VOCl_2$ at certain concentrations, is definitely beneficial for growth in *Chilomonas* and that the optimum concentration is between 10^{-5} M and 7×10^{-5} M.

In some of the experiments which were continued for shorter periods the concentration of $VOCl_2$ was lower than 10^{-7} M and in others it ranged from 10^{-7} M to 10^{-4} M. No verifiable acceleration of growth was obtained in the experiments with concentrations lower than 10^{-7} M, but definite acceleration was obtained at approximately 10^{-5} M in the other experiments.

TABLE III

Effect of oxidized vanadium (pentavalent), in Na_3VO_4, in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature, 24 ± 0.5° C.

Molar Concentrations of Na_3VO_4	Average Number Divisions per Day of Four Lines (15 days)
0	3.76±.063*
10^{-12}	3.74±.057
10^{-10}	3.68±.054
10^{-8}	3.59±.049
10^{-7}	3.73±.045
10^{-6}	3.52±.047
10^{-5}	3.65±.051
10^{-4}	3.63±.052
10^{-3}	3.50±.044

* Probable errors.

Table III shows that there was no increase in rate of reproduction of *Chilomonas* in acetate-ammonium solution containing oxidized vanadium (pentavalent), in Na_3VO_4, but that in the highest concentration tested the rate of reproduction was considerably lower than in the

TABLE IV

Effect of Na_3VO_4 and NaCl in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature 24 ± 0.5° C.

Molar Concentrations of Sodium	Average Number Divisions per Day of Four Lines (7 days)	
	Na_3VO_4	NaCl
0	3.34±.067*	3.34±.067*
3×10^{-3}	3.00±.093	3.28±.060
3×10^{-7}	3.35±.082	3.37±.071

* Probable errors.

acetate-ammonium solution. This experiment was repeated several times. Essentially the same results were obtained in all. Since a molecule of Na_3VO_4 contains three atoms of sodium and only one of vanadium, the question arises whether the inhibition in the rate of growth was due to the sodium or to the vanadium. To answer this question *Chilomonas* was grown in acetate-ammonium solution and in this solution which contained respectively Na_3VO_4 and NaCl in such quantities that the concentration of sodium was the same in both. The results obtained are given in Table IV.

Table IV shows that during seven successive days the average

number of divisions per day in acetate-ammonium solution containing 10^{-3} M Na_3VO_4 was 0.34 of a division less than in the acetate-ammonium solution with no Na_3VO_4 or NaCl, and that in the solution containing 3×10^{-3} M NaCl the average was only 0.06 of a division less. It also shows that the frequency of division in the solution containing 10^{-7} M Na_3VO_4 and in that containing 3×10^{-7} M NaCl was essentially the same as in the acetate-ammonium solution. These results demonstrate therefore that the decrease in the rate of growth in 10^{-3} M Na_3VO_4 was due to the vanadium and not to the sodium in it. This conclusion is supported by the rate of reproduction in 3×10^{-4} M $VOCl_2$ (Table II).

Discussion

The fact that *Chilomonas* can tolerate Na_3VO_4 in concentrations as high as 10^{-3} M indicates that vanadium in this form is not toxic for protozoa. This conclusion is supported by the results obtained by Proescher and Seil (1917). They found that *Opalina ranarum* can tolerate concentrations of $Na_2V_4O_{11}$ as high as 0.284 M.

In other organisms vanadium has been found to be beneficial or detrimental depending on the concentrations. Suzuki (1903) showed that low concentrations of VSO_4 cause increase in the rate of growth of the roots of barley plants but not the total growth and that high concentrations cause cessation of growth. Scharrer and Schropp (Willis, 1936) found that in high concentrations of $NaVO_3 \cdot H_2O$ plant growth is retarded, if not completely stopped, but that in low concentrations there are indications of acceleration, and Shibuya and Saeki (Willis, 1936) report acceleration of plant metabolism by vanadates. Proescher and Seil (1917) report only injurious effects of vanadium on wheat seedlings.

Konishi and Tsuge (1933) studied the elements in soils in which the nitrogen-fixing bacterium *Azotobacter chroococcum* grows. They found by spectroanalysis that vanadium is always present in soil in which *Azotobacter* makes good growth, and that if either VCl_2 or $NaVO_3$ is added in concentrations of either 10^{-4} or 10^{-5} M to an artificial culture medium the amount of nitrogen fixed is five to ten-fold greater than that fixed in culture medium containing no vanadium.

Copper

According to Elvehjem (1935) Meissner first demonstrated, in 1817, that copper occurs in plants. It was not until about a century later that its general occurrence in plants was established by Guerithault (1920), Maquenne and Demoussy (1920) and others. These workers found considerable amounts in fresh plant tissues and seeds. Ma-

quenne and Demoussy found it in so many plants that they suggested that it must be essential in plant growth. Since then numerous investigators (Felix, 1927; Allison, Bryan and Hunter, 1927; Orth, Wickwire and Burge, 1934) have demonstrated that many soils are improved for plant growth by the addition of $CuSO_4$, and Sommer (1931) demonstrated that the element is necessary for plant growth. More recently, Kubowitz (1937) has shown that the polyphenol oxidase of the potato is a copper-containing protein. This is a possible explanation of the necessity of copper for plant growth.

Raulin (1869) believed that probably other metals besides iron and zinc are necessary for the growth of *Aspergillus niger*. Ono (1902) confirmed this belief when he obtained greater dry weights of *Aspergillus niger* by adding small amounts of copper to the culture solution. Lepierre (1913) maintains that zinc in Raulin's solution can be replaced, at least partially, by copper.

Up until recent years only two natural occurrences of copper in animals were known—in hemocyanin and in turacin, the red pigment of the feathers of the Turaco birds of South Africa. Thudichum (1901) claimed that he found it in the human brain, but his claim was not accepted until Bodansky (1921) found considerable amounts in the brains of four people. Now, because Hart, Steenbock, Waddell and Elvehjem (1928) demonstrated that highly purified iron salts are effective in correcting a deficiency of hemoglobin only when small amounts of copper are present, it is recognized that it plays an important rôle in the life of mammals.

Experimental Procedure

The effect of copper in $CuCl_2$ on the growth of *Chilomonas* was tested. Acetate-ammonium solution containing $CuCl_2$ in various concentrations was prepared, the hydrogen ion concentration adjusted and maintained, the solutions sterilized and the experiments made as described above. The results obtained are given in Table V.

Results

By referring to Table V it will be seen that in acetate-ammonium solution containing concentrations of $CuCl_2$ of 10^{-12} M and less, the frequency of division was essentially the same as in acetate-ammonium solution with no copper (3.76 divisions per day) and that in concentrations higher than 10^{-12} M the frequency of division decreased as the concentration of $CuCl_2$ increased, division ceasing entirely at 3×10^{-7} M. In 3×10^{-7} M the chilomonads divided and lived five or six days. As the concentration increased from this the length of life decreased until at 10^{-6} M they lived only a few minutes.

Discussion

The results concerning the toxicity of copper are in accord with those obtained by others. Nägeli (1893) found that copper kills algae in extremely low concentrations. Seybold (1927) found that *Euglena* lives in 10^{-8} M $CuSO_4$ as long as food lasts, but that in 10^{-7} M it dies in less than half an hour.

Chalkley and Voegtlin (1932) found that $CuCl_2$ in a concentration as low as 2×10^{-8} M kills *Amoeba proteus* and that 2×10^{-9} M depresses growth, increases the rate of mortality, and probably decreases the rate at which food in the food vacuoles is digested and assimilated.

TABLE V

Effect of copper in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature $24 \pm 0.5°$ C.

Molar Concentrations $CuCl_2$	Average Number Divisions per Day of Four Lines (15 days)
0	3.76±.046*
10^{-20}	3.70±.056
10^{-18}	3.74±.062
10^{-16}	3.76±.051
10^{-14}	3.76±.051
10^{-12}	3.79±.045
10^{-10}	3.60±.056
10^{-8}	3.42±.072
10^{-7}	3.24±.088
3×10^{-7}	died
5×10^{-7}	"
8×10^{-7}	"
10^{-6}	"

* Probable errors.

Sommer (1931) found that flax, sunflower and tomato plants mature normally when grown in artificial nutrient medium containing copper but do not if it does not contain copper, and Ono (1902), Bortels (1927) and Roberg (1928) showed that small amounts of copper cause an increase in the total growth of *Aspergillus niger*. This indicates that copper is beneficial for growth in many different organisms.

The fact that the rate of reproduction in *Chilomonas* was no higher in any of the concentrations of copper tested than in acetate-ammonium solution without copper indicates that copper is not beneficial for reproduction in this form. The facts that it has been grown for eight years in this laboratory in solutions composed of chemicals of the highest purity with no copper added and that the water in these

solutions was triply distilled in a tandem Pyrex glass still and was kept in Pyrex glass containers, i.e. in glass which according to the makers contains no copper (communication from the Corning Glass works) strongly indicate that copper is not needed for growth in *Chilomonas*.

Manganese and Iron

McHargue (1926) says that in 1788 Scheele showed that pyrolusite (MnO_2) is the oxide of manganese which was then a new metal and that manganese is assimilated by plants. Manganese has since been found in so many plants by Maumené (1884), Headden (1915) and Bertrand and Rosenblatt (1921) that it is now believed to be in all (Miller, 1931).

The addition of manganese to soil was early found to be beneficial to the growth of plants (Nagaoka, 1903; Bertrand, 1905; Brenchley, 1910; McHargue, 1926; and Bryan, 1929). Bertrand (1905) obtained such poor growth in plants with deficiencies of it that he suspected it to be indispensable for growth. This was proved to be true by McHargue (1926), Gilbert and Pember (1931), and McHargue and Calfee (1932).

According to Oettingen (1935) manganese has been found in the blood, the liver and the kidneys in relatively large quantities and in lesser quantities in all other tissues in animals. Its functional rôle in animals is, however, not definitely known. Some investigators (Titus, Cave and Hughes, 1928; Myers and Beard, 1931) found it beneficial in blood regeneration and others (Waddell, Steenboch and Hart, 1929; Orent and McCollum, 1931; and Mitchell and Miller, 1931) found it either non-beneficial or only negligibly beneficial. Orent and McCollum found that rats fed on a diet free of manganese grow to maturity in an apparently normal manner, but that their offspring are undersized and inferior in appearance and that they are not suckled by their mothers. They also found that male rats raised on such a diet develop normally but have degenerated testes.

Gris discovered in 1845 that iron is a constituent of chlorophyll-bearing plants (Bortels, 1927). Since then it has been established that green plants which are deprived of it become chlorotic due to the absence of chlorophyll (Miller, 1931). Raulin (1869) found that if iron is added to culture media in which *Aspergillus niger* is growing the total weight produced is greatly increased.

Iron is found in nearly all if not all animal tissues. It is in all red blood corpuscles and Jones (1920) found it in the nuclei of cells of the liver and kidney and in the cytoplasm and the nuclei of cells of the spleen in adult and foetal guinea pigs. He found it in various tissues of the sparrow, frog, fish, crayfish, oyster, earthworm and

hydra, and says that staining reactions for it are stronger in lower animals than in higher and in foetal mammalian tissues than in adult.

It is well known that iron is closely associated with the transfer of oxygen by blood. In plants it is thought to act catalytically in the formation of chlorophyll (Wolff, 1913). Warburg (1925) contends that in all cells it functions mainly in respiration and that this is due to the catalytic action of a hematin derivative.

Experimental Procedure

The effect of manganese in $MnCl_2$ and iron in $FeCl_3$ on the growth of *Chilomonas* was tested in accord with the preceding experiments on vanadium and copper. The results obtained are presented in Table VI.

TABLE VI

Effect of manganese and iron in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction of *Chilomonas paramecium*. Temperature 24 ± 0.5° C.

$MnCl_2$ and $FeCl_3$ Added; Molar Concentrations	Average Number Divisions per Day of Four Lines	
	$MnCl_2$ (19 days)	$FeCl_3$ (17 days)
0	3.28±.055*	3.30±.045*
10^{-9}	3.25±.045	3.38±.046
5×10^{-9}	3.17±.063	3.37±.045
10^{-8}	3.31±.058	3.37±.041
5×10^{-8}	3.39±.049	3.40±.045
10^{-7}	3.33±.048	3.33±.049
5×10^{-7}	3.40±.046	3.39±.042
10^{-6}	3.25±.041	3.33±.057
5×10^{-6}	3.28±.051	3.47±.045
10^{-5}	3.38±.042	not tested
5×10^{-5}	3.28±.049	" "
10^{-4}	3.39±.041	" "

* Probable errors.

Results

Table VI shows that in the solutions to which $MnCl_2$ was added the frequency of division varied from 3.17 to 3.40 per day and that in those to which $FeCl_3$ was added it varied from 3.33 to 3.47 per day, but that there is no indication of any correlation between the concentration of either manganese or iron and the frequency of division and no statistically significant difference between the rate of reproduction in the solutions to which iron and manganese were added and the acetate-ammonium solution. These tests were repeated and essentially the same results were obtained. They therefore show that iron and manganese neither augment nor retard growth in *Chilomonas*.

Cultures of *Chilomonas* have been maintained in this laboratory in acetate-ammonium solution for nearly eight years without any indication of loss of vigor. Neither iron nor manganese was added to the solution. It consequently appears that neither is necessary for growth in *Chilomonas*. Kahlbaum maintains, however, that the $CaCl_2$ used in the solution contains 0.0001 per cent iron and some of the salts used may have contained a trace of manganese. The fact that *Chilomonas* grows in acetate-ammonium solution does not therefore prove that iron and manganese are unnecessary, but the fact that neither was detected in the tests described above which are sensitive to 5×10^{-8} M for iron and 10^{-8} M for manganese demonstrates conclusively that if these metals are necessary extremely minute quantities suffice.

Discussion

Molisch (1894) grew *Aspergillus niger* and *Penicillium* sp. in a culture medium to which no iron was added. However, analysis showed that they contained iron, and when iron was added to the culture medium the amount of growth was much greater. Molisch therefore concluded that these two molds need iron. The conclusion that *Aspergillus* needs iron has been amply verified by Sauton (1911), Steinberg (1935), Bortels (1927) and Roberg (1928). Sauton also found that *Penicillium* needs iron, but that the molds *Mucor mucedo*, *Rhizopus nigricans* and *Racoelium coellare* do not need it.

Bertrand and Javillier (1911) found that the dry weight of *Aspergillus niger* increased as the manganese content increased to 1000 mg. per 100 cc. and then decreased. Later Bertrand (1912) obtained augmentation of a crop of mold by the addition of an amount of manganese equivalent to one mg. per 10,000 liters of nutrient medium and because of this evidence suggested a catalytic rôle of the metal.

Hotchkiss (1923) found that iron does not accelerate the growth of *Bacterium coli*, although it has been found to accelerate the growth of many bacteria. Koser, Finkle, Dorfman and Saunders (1938) tested the possibility that inorganic salts present as impurities in preparations of spleen, liver, and yeast cause the growth-promoting activity of these preparations. They found that salts of neither iron, manganese nor copper were responsible for the growth in five bacteria and a yeast caused by additions of the preparations; however, they point out that their results do not unequivocally invalidate the evidence of the numerous investigators who have found that small amounts of the heavy metals do accelerate cell reproduction.

Hall (1937) maintains that manganese causes acceleration in growth in *Euglena anabaena*, but not in *Colpidium campylum* and *Astasia*.

These studies show that, although the heavy metals cause acceleration of growth in many organisms, they do not do so in all, and they seem to be indispensable to some forms and not to others. As already stated, the results of Tables V and VI show that the growth of *Chilomonas* is not accelerated by additions of copper, manganese and iron, and they indicate that these metals are not indispensable to *Chilomonas*. More evidence, however, is highly desirable.

Some evidence concerning the necessity of iron is found in the results obtained in the study of respiration in *Chilomonas*. If *Chilomonas* respires by means of an enzymatic iron-porphyrin system, the most common of which is that involving cytochrome and indophenol oxidase, iron is obviously necessary. Cytochrome, a hemochromogen, and indophenol oxidase are found in most aerobic organisms and tissues, including mammalian tissues, plants, insects, yeasts and aerobic bacteria (Meldrum, 1934). However, tests of a heavy suspension of chilomonads by means of a hand spectroscope and a microspectro-

FIG. 1. Camera outlines showing the variation in starch and fat content in specimens of *Chilomonas paramecium* in acetate-ammonium solution (Table I) and in this solution with vanadium (5×10^{-4}M), copper (10^{-10}M), manganese (5×10^{-5}M) and iron (10^{-5}M) added respectively. ○, starch; ●, fat. Note that the starch and fat content varied from no starch and much fat to much starch and little fat and that this occurred in specimens grown in acetate-ammonium solution as well as in this solution with metals added.

scope revealed no absorption bands characteristic of cytochrome, and Hutchens (1939), working in this laboratory, reports that the respiratory system of *Chilomonas* is not sensitive to cyanide, which indicates the absence of indophenol oxidase and cytochrome.[2]

Relation between Vanadium, Copper, Manganese and Iron and the Amount of Starch and Fat in Chilomonas

From time to time chilomonads grown in acetate-ammonium solution and in this solution containing the various metals under consideration were studied under the microscope to ascertain the effect of the metals on the starch and fat content. This was done as follows:

[2] Since this paper went to press, Hutchens, by personal communication, has reported the presence of cytochrome and a sensitivity of respiration to cyanide in *Chilomonas* when grown in a more complex medium in which greater numbers of organisms are obtained.

Equal numbers of specimens were selected at random from each of the four lines in a given solution and put into a vaseline ring on a glass slide with as little water as possible; then lugol solution was added and a few minutes later sudan III. This fixed the chilomonads and stained the starch and fat in them. Specimens representing the extent of variation in fat and starch content were then selected and camera outlines made. One of the specimens in each solution which contained the greatest amount of starch and one which contained the greatest amount of fat are presented in Fig. 1.

This figure shows that the relative content of starch and fat varied enormously in different specimens, but that it varied just as much in those grown in acetate-ammonium solution as in those grown in this solution containing any one of the metals used and that there is no correlation between this variation and the kind or the concentration of these metals.

Observations on the relation between the rate of reproduction of the chilomonads in these solutions and the starch and fat content show that the fat content varied inversely and the starch content directly with the rate of reproduction in all of the solutions, the acetate-ammonium solution as well as this solution plus the metals. The relative amount of starch and fat in *Chilomonas* is therefore correlated with the rate of reproduction.

Summary

1. Experiments were performed to ascertain the effect of vanadium ($VOCl_2$ and Na_3VO_4), copper ($CuCl_2$), manganese ($MnCl_2$) and iron ($FeCl_3$) in acetate-ammonium solution on the rate of reproduction and the synthesis of starch and fat in *Chilomonas paramecium*.

2. Tetravalent vanadium in $VOCl_2$ causes marked increases in the frequency of division in *Chilomonas* which vary with the concentration. As the concentration of $VOCl_2$ added to the acetate-ammonium solution was increased, the frequency of division increased to a maximum at approximately 10^{-5} M and then decreased. Pentavalent vanadium in Na_3VO_4 causes no increase in frequency of division. Tetravalent vanadium at certain concentrations is therefore beneficial for growth of *Chilomonas* and pentavalent vanadium is not.

3. Neither copper, manganese nor iron in acetate-ammonium solution causes any statistically significant increase in the frequency of division of *Chilomonas*, and neither manganese nor iron causes a significant decrease in the frequency of division, but as the concentration of copper increases from 10^{-12} M the frequency of division decreases, ceasing entirely at 3×10^{-7} M.

4. *Chilomonas* has been grown continuously for eight years in acetate-ammonium solution with no additions of copper, manganese or iron. This shows conclusively that if the metals are needed extremely minute quantities suffice, but it does not prove that they are unnecessary for there are doubtlessly traces of them in the solution.

5. The starch and fat content of *Chilomonas* varied from no starch and much fat to much starch and little fat in all the solutions used; therefore, this variation was not caused by the addition of either vanadium, copper, manganese or iron. It was found in all solutions to be correlated with the rate of reproduction.

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VITA OF WILLIAM JONES BOWEN

William Jones Bowen was born in Wallace, New York, County of Steuben, on September 14, 1911. His primary education was obtained at the Wallace Graded School and his secondary at Avoca High School, Avoca, New York, and at Haverling High School, Bath, New York.

In September, 1930, he matriculated at the University of Buffalo, Buffalo, New York, and from this institution received the degree of Bachelor of Arts, cum laude, in 1934. During the years 1932–33 and 1933–34 he was student assistant in the laboratory of Comparative Anatomy and did research work under the direction of Professor William L. Dolley under the Tutorial Plan of the University of Buffalo. The research concerned the reactions to varying intensities of light of the drone fly, *Eristalis tenax*. During the year 1934–35 he continued these studies and received the degree of Master of Arts in 1935. The title of his thesis for the Master's degree is "The effect of age and of sex on dark adaptation in the eye of *Eristalis tenax*."

In September, 1935, he enrolled as a graduate student in the Department of Zoology of the Johns Hopkins University where he held a University Scholarship in 1935–36. During the years 1936–37 and 1937–38 he held the graduate assistantship in General Physiology. During the summer of 1936 he was at the Marine Biological Laboratory at Woods Hole, Massachusetts, where, with the aid of a scholarship from the Johns Hopkins University, he was a student in Invertebrate Zoology. The year 1936–37, the summer of 1937, and the year 1937–38 he did research for his doctor's dissertation.

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